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UPTAKE OF AMINO ACIDS BY MARINE POLYCHAETES UNDER ANOXIC CONDITIONS

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Representative species of at least twelve different families of the class Polychaeta have served as experimental organisms in studies of transepidermal transport of small organic solutes in several laboratories. These studies include many published reports dealing with specific aspects of this phenomenon (reviewed by Stephens, 1972; Jørgensen, 1976) as well as unpublished work in this laboratory. Since marine annelids are often conspicuous members of the infauna, are easy to collect and maintain for brief periods in the laboratory, and live in habitats relatively rich in dissolved organic resources, they continue to be used for such work (Ahearn and Gomme, 1975; Rice and Chien, 1978; Stephens, 1975). Despite this attention, basic characteristics of this influx remain unexamined. We undertake to explore two aspects of influx of amino acids in two representative polychaetes.

First, the relation between influx of labeled substrates and net movement of substrate into or out of the animal is still in question. Stephens (1975) showed net influx of known amino acids in *Nereis diversicolor* and *Capitella* sp. using a fluorometric procedure to follow disappearance of added substrate. However, labeled substrates were not employed in this work. Details of the relation between influx of substrate followed radiochemically and net movement of substrate followed chemically have been described and analyzed for molluscs (Wright and Stephens, 1977, 1978; Crowe, Dickson, Otto, Colon and Farley, 1977) and for enchinoderms (Stephens, Volk, Wright and Backlund, 1978). The only report concerning annelids is that of Johannes and Webb (1970) in which they describe influx of ^{14}C-labeled glycine accompanied by net efflux of amino acids in *Clymenella*. Since many of the reports concerning influx of ^{14}C-labeled substrates overtly or tacitly assume that such influx represents net removal of substrate from the medium by the animal and draw conclusions on this basis, the subject merits investigation.

Second, infaunal polychaetes undergo periods of relative oxygen deprivation or anaerobiosis. If net influx of small organic molecules is to be invoked as a potential supplementary form of nutrition for these animals, the response of the process to relative or complete anoxia is important.

Stephens (1963) reported that influx of ^{14}C-labeled amino acids in *Clymenella* was unimpaired after long exposure to sea water through which gaseous N_2 was bubbled. However, no direct measurements of P_{O_2} were made. Several authors have used inhibitors of aerobic metabolism and reported partial inhibition of influx rates in some cases, and no clear response in other cases. None of this work includes independent criteria to assess inhibitor effects, and in most cases does not demonstrate reversibility of effects reported.

The present work reports the relation between influx and net flux of glycine in two genera of polychaetes. This relation is reported over a range of P_{O_2} including

anoxic conditions. The effects of anoxia are compared to effects of cyanide inhibition of aerobic metabolism.

Materials and Methods

Specimens of *Marphysa sanguinea* (Eunicidae) were collected intertidally from Upper Newport Bay, Newport Beach, California. Animals were found in muddy sediment under and between intertidal rocks. Specimens of *Pareurythöe californica* (Amphinomidae) were collected subtidally from a shallow lagoon at Point Mugu Naval Base near Port Hueneme, California. The animals occurred in sandy sediment just below mean low tide. Animals were maintained in the laboratory in aerated containers at 16° C. Specimens of both genera were selected in the wet weight range of 0.7 to 1.4 g for observations. Experiments were carried out at room temperature (23° C); animals were acclimated to the temperature change for several hours before use.

Influx of ^{14}C-glycine into animals was followed by periodic sampling of the medium in which animals were incubated. Experimental media were prepared from ^{14}C-gly (UL) at 20 to 40 μCi/liter plus sufficient ^{12}C-gly to achieve the desired chemical concentration. All media were prepared in artificial sea water (MBL) prepared according to Cavanaugh (1956) filtered through a Millipore filter (0.45-μm pore size). Duplicate 0.2-ml samples of medium were taken periodically, acidified to drive off CO_2, and added to a toluene-based scintillation cocktail containing a detergent. Radioactivity was determined using a Beckman CPM-100 scintillation counter. Details of sampling protocol vary according to the experimental procedure.

Net change in ambient glycine concentration was followed using fluorescamine to determine changes in primary amines in solutions in which animals were incubated. The procedure has been described (Stephens, 1975; Stephens *et al.*, 1978). The reagent, fluorescamine, reacts with primary amines to produce a fluorescent product with an absorption maximum at 390 nm and emission peak at 480 nm. Fluorescence was measured using a Perkin-Elmer spectrophotofluorometer. Initially, fluorescence reflects glycine concentration since glycine is the only primary amine present in the medium. After incubation, fluorescence represents remaining glycine plus any primary amines which may be present as a result of efflux from the animal. Fluorescence is expressed in units of equivalent glycine concentration.

Influx and net flux of glycine under anaerobic conditions was measured as follows: Several hundred ml of MBL sea water was placed in a large culture flask with a port at the base in which an oxygen electrode (YSI) was mounted. Nitrogen gas was passed through acid pyrogallol and bubbled through the culture flask using a breaker stone. Oxygen content was monitored with the oxygen meter until anoxic conditions were achieved; approximately 5 min were required to reach the same oxygen reading as that obtained for sea water chemically deoxygenated with dithionite. Four samples of 50 ml each were then siphoned into four flasks, each containing one worm. Two of these flasks were placed in the N_2 gas train and maintained anoxic. The other two were reoxygenated using an air pump and served as controls. Worms were allowed to adapt to these conditions

TABLE I

Rates of uptake of glycine in specimens of Pareurythöe *and* Marphysa *under aerobic and anaerobic conditions. In all cases, initial concentration of glycine was* 20 μM. *Influx was calculated from the rate of depletion of* ^{14}C-*glycine from the medium; net influx refers to the rate of disappearance of total primary amines from the medium, and is expressed in terms of glycine-equivalents.*

Experimental condition	Influx [moles × 10^{-7}/(g·hr)]	Net influx [moles × 10^{-7}/(g·hr)]
Pareurythöe		
Anaerobic	2.9	3.2
	3.0	3.2
	2.0	1.3
	2.5	2.3
	Average 2.6 ± 0.3 (s.d.)	2.5 ± 0.8
Aerobic	5.4	5.7
	4.6	5.2
	8.9	8.2
	7.8	8.0
	Average 6.7 ± 1.7	6.8 ± 1.3
Marphysa		
Anaerobic	2.2	—
	1.9	—
	1.2	1.2
	3.1	3.3
	Average 2.1 ± 0.7	2.3
Aerobic	7.4	—
	8.5	—
	4.9	4.9
	5.2	4.9
	Average 6.5 ± 1.5	4.9

for 15 to 30 min. Substrate (0.5 ml) was then added using a hypodermic syringe to initiate the experimental period. Samples were withdrawn periodically using a hypodermic syringe and were used for duplicate determinations of radioactivity and duplicate determinations of fluorescence at each time point.

Observations on influx and net flux of glycine in *Pareurythöe* were also carried out using a flow system. Filtered MBL sea water containing ^{14}C-labeled glycine (20 μm) was placed in a flask to serve as a medium reservoir. A metering pump was used to produce a flow of medium through a chamber with sintered glass discs at either end (internal diameter = 16 mm, length = 75 mm, approximate volume = 15 ml). Medium from the reservoir was led to the chamber via a sampling port which permitted monitoring P_{O_2} as it passed into the chamber. The medium reservoir was initially deoxygenated and then reoxygenated to the desired level. By using a relatively large volume of medium in the reservoir with a small free surface, negligible drift in P_{O_2} was encountered during the course of observations. Flow rate was calculated to be sufficiently rapid that oxygen consumed by the worm would represent a depletion of 10% or less of the oxygen content of sea water in equilibrium with air. This represents a necessary compromise which allowed for a minimal flow rate sufficient to permit reliable determinations of the difference between inflow and outflow concentrations. Actual flow rates ranged from 3 to 6 ml/min depending on the weight of the worm. At

each level of P_{O_2} tested, medium was pumped through the chamber for 15 min prior to sampling. Samples were then taken each 5 min for the following 30 min and analyzed in duplicate for radioactivity as described. In some experiments the fluorescence at inflow and outflow ports was also determined.

In all cases where medium depletion was followed with time, a straight line was obtained when log radioactivity or log fluorescence was plotted against time, indicating that depletion followed first order exponential kinetics. Uptake rates are presented as moles glycine/(g wet weight·hour) from an ambient concentration of 20 μM. Data from the first hour of sampling was used to establish the rate constant. Uptake rates obtained using the flow system were calculated from the average difference between inflow and outflow samples as described and are expressed in the same units.

Results

Table I presents rates of influx and net rates of influx for *Pareurythöe* and *Marphysa* under anaerobic and aerobic conditions. There is no significant difference

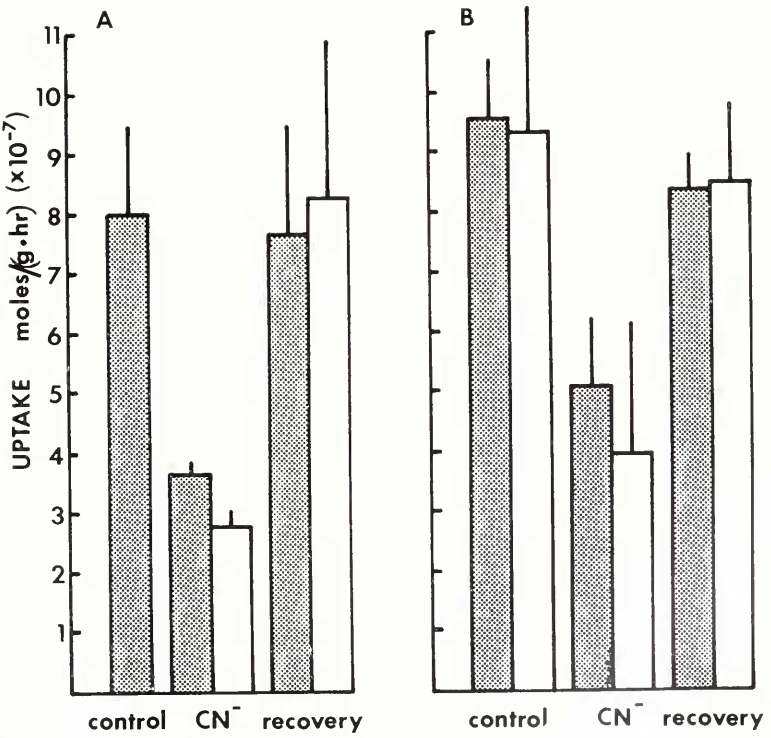

Figure 1. Effect of cyanide on the influx and net influx of glycine into A) *Marphysa* and B) *Pareurythöe*. Influx (shaded bars) was calculated from the rate of depletion of ^{14}C-glycine from the experimental medium; net influx (open bars) was determined from the rate of depletion of total primary amines. In all cases the initial concentration of glycine was 20 μM; the concentration of KCN was 2 mM. Bars represent +1 standard deviation.

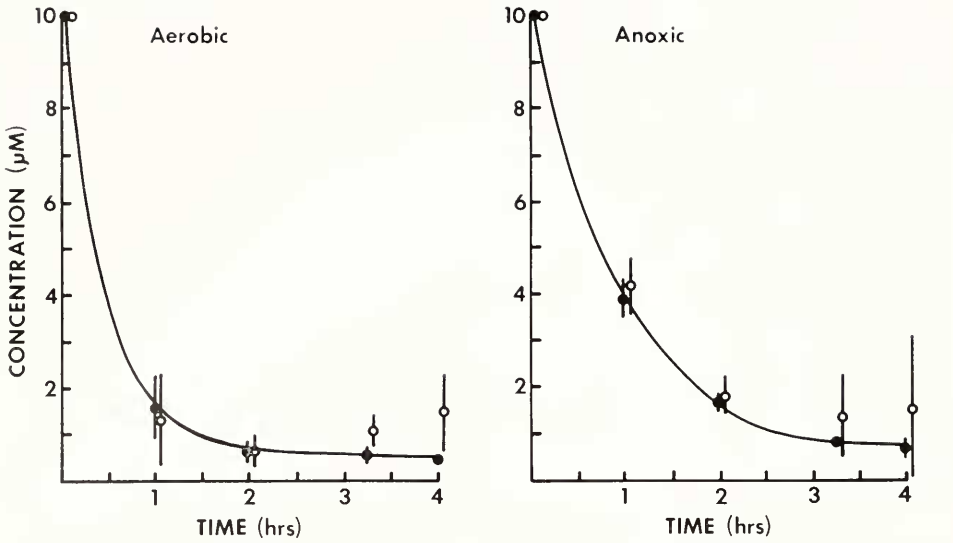

FIGURE 2. Effect of anoxia on the time course of influx and net influx of glycine into *Pareurythöe*. Solid circles represent measured levels of ^{14}C-glycine; open circles are concentrations of total primary amines expressed as equivalent glycine concentration. Anoxic conditions were produced by bubbling N_2 through experimental media containing 2 mM KCN. Each point is the mean of three separate determinations with individual worms; bars represent ± 1 s.d..

between rates of influx (^{14}C depletion) and net influx (decrease in primary amines). Hence there is a net transfer of substrate from the medium to the animal at a rate accurately estimated by either procedure. Rates of uptake under anaerobic conditions are reduced to levels 32 to 46% of aerobic rates.

Figure 1 presents data for influx and net influx of glycine in the presence and absence of 2 mM KCN. Oxygen consumption for three individuals of *Marphysa* was measured using an oxygen electrode. Exposure to 2 mM KCN for 30 min inhibited oxygen consumption to 13% of control values. Normal rates of oxygen consumption were restored when animals were permitted to recover for 24 hr. Influx and net flux of glycine into three individuals of each species before, during and after exposure to 2 mM KCN was measured on two separate occasions. Influx and net flux were reduced in the presence of cyanide to levels essentially the same as those observed under anaerobiosis (40 and 46% of control values). Recovery was complete.

Figure 2 presents data for *Pareurythöe* showing the time course of influx of ^{14}C-glycine and net flux of total primary amines under aerobic and anoxic conditions. The data for each experimental condition are mean values of separate studies on three worms. Anoxic conditions were produced by bubbling N_2 through experimental media also containing 2 mM KCN. Similar results were obtained using *Marphysa*. Note the close correspondence between influx as determined by depletion of radioactivity and net disappearance of substrate as indicated by determination of fluorescence. This is the case under both sets of conditions. In

both cases, determinations of fluorescence begin to diverge from determinations of radioactivity after two hours. In both cases, radioactivity does not continue to decline indefinitely. This does not represent a limitation of the transport system since ^{14}C-glycine at concentrations of 2 to 4×10^{-7} M is removed exponentially by both species.

Figure 3 presents data for two individuals of *Parcurythöe* in which influx and net influx of glycine was measured in the flow system. Error bars represent ±1 standard deviation of the average difference of seven determinations of radioactivity or fluorescence at inflow and outflow ports. Actual differences range from 0.6 to 7% of inflow. Since P_{O_2} was measured at the inflow, the values are systematic overestimates of the average P_{O_2} experienced by the animal during the observation period. Six such experiments were performed. In all cases there was a sustained high rate of influx, essentially comparable to that observed in aerobic depletion experiments, until ambient P_{O_2} was reduced to approximately 10% to 20% of air saturation values. In some cases, there was some decrease with decreasing P_{O_2} prior to the more conspicuous decrease observed at very low P_{O_2}. The data are too variable to justify mathematical treatment but suffice to demonstrate that influx and net influx are not linearly related to P_{O_2} over this range of oxygen concentrations. In these observations, influx and net influx of glycine were inhibited to a greater extent under anoxic conditions than the inhibition

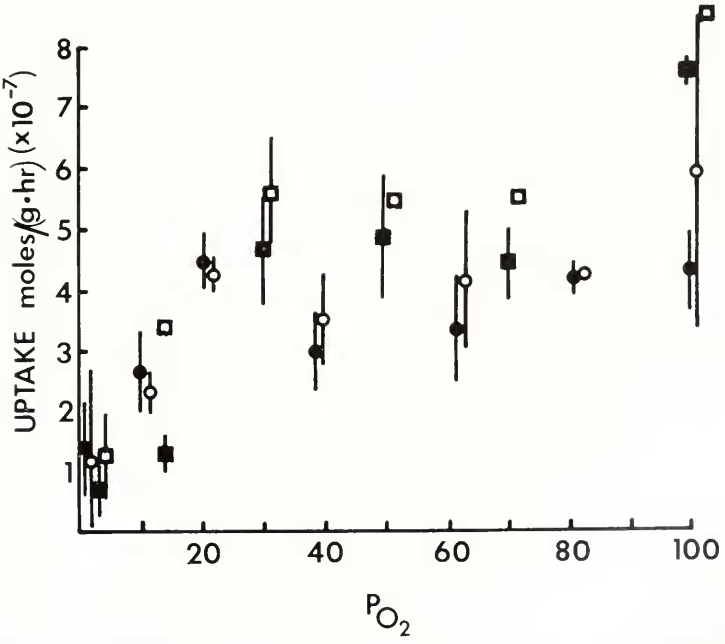

FIGURE 3. Effect of P_{O_2} on influx and net influx of glycine into *Parcurythöe*. Circles and squares represent separate experiments on two individual worms. Closed symbols represent influx of ^{14}C-glycine; open symbols represent net influx of total primary amines. Bars are ±1 s.d.; those cases in which open symbols have no error bars indicate that the variability was too large to include effectively in the figure.

TABLE II

Nutritional role of amino acid uptake in Pareurythöe *and* Marphysa: *examination of critical parameters. See text for the basis of the calculations.*

	Pareurythöe	*Marphysa*
Concentration of free amino acids in environment (μM)	123	131
Oxygen consumption [ml/(g·hr)]	0.13	0.15
Influx [moles × 10^{-7}/(g·hr)] (*cf.* Table I, for 20 μM)	6.7	6.9
Contribution for 20 μM gly (μg)	50.5	44.6
% oxygen requirement	17.4	13.8
Glycine concentration for 100% oxygen requirement (μM)	115	145
Mixed amino acid concentration for 100% oxygen requirement (μM)	52	65

observed in depletion experiments. Anaerobic influx rates were approximately 15% to 20% of those observed at higher P_{O_2}.

DISCUSSION

There is no significant difference between rates of ^{14}C-glycine influx in *Marphysa* and *Pareurythöe* estimated from radiochemical measurements and rates of net influx of glycine estimated from fluorometric determinations from an ambient glycine concentration greater than 10 μM (Figure 2, Table I). There is a slow efflux of primary amines (fluorescamine-positive material) with time in both aerobic and anoxic conditions (Figure 2). These findings agree with similar studies of molluscs (Wright and Stephens, 1977, 1978) and echinoderms (Stephens *et al.*, 1978). In all of the soft-bodied marine invertebrates examined adequately, influx of ^{14}C-labeled amino acids reflects net influx quite accurately at concentrations greater than 10 μM; however, this process is accompanied by a slow efflux of primary amines.

These results agree with the reports of Johannes, Coward and Webb (1969) and Johannes and Webb (1970) using the flatworm, *Bdelloura candida* and the polychaete, *Clymenella*. They supplied labeled substrates at low concentrations (0.6 μM and 1.0 μM in the studies cited) and observed a slow net efflux of amino acids with time at rates broadly comparable to those reported here and those reported by Stephens (1968). However, they interpreted their data as evidence for exchange diffusion and questioned the occurrence of net influx of substrate into marine invertebrates, suggesting that earlier literature might represent a misinterpretation of such exchange diffusion. However, exchange diffusion is clearly excluded as a mechanism of efflux by data showing that efflux is essentially independent of ambient concentrations, as appears to be the case in the examples previously cited. Beyond this, there is currently no evidence concerning the route or mechanism of efflux.

Table II presents measurements of naturally occurring primary amines in the immediate habitat of *Pareurythöe*. Data were obtained by expressing interstitial water from freshly collected sediment cores in the sandy subtidal habitat of *Pareurythöe*. Total free amino acids were estimated by the fluorescamine technique. Some of these data are reported in Stephens *et al.* (1978) where methodological details are presented. The samples ranged from 54 to 244 μM (average 123 ± 54

μM, n = 13). Free amino acids in the habitat of *Marphysa* were estimated in a similar fashion. However, it was not possible to obtain cores in the immediate area of the rocks where the animals were found. Therefore, cores were taken from otherwise comparable muddy sediment in immediately adjacent areas of Newport Bay. Average free amino acids ranged from 88 to 180 μM (average 131 $\pm$ 35 μM, n = 7). Table II also presents data for oxygen consumption of *Pareurythöe* in the size range employed in these experiments [0.13 ml/(g·hr) $\pm$ 0.02, n = 6]. For *Marphysa,* oxygen consumption was 0.145 $\pm$ 0.25 (n = 8). Table II also includes average rates of glycine influx (average of influx and net influx from Table I) for the two worms. Given these data, it is possible to calculate numbers to estimate the significance of amino acid influx as compared to metabolic requirements estimated from oxygen consumption. Complete oxidation of 1.0 mg of glycine requires 2.23 ml O_2 (STP). One can calculate the percentage contribution of glycine influx from an ambient concentration of 20 μM (where measurements were made) for the two cases. By making the assumption that influx at higher concentrations is linearly related to concentration, the glycine concentration in the medium which would provide sufficient reduced carbon to account for oxidative metabolism can be calculated. In fact, interstitial amino acids from the immediate habitat of *Pareurythöe* have been identified chromatographically (Stephens *et al.,* 1978). Glycine is present as well as seven other identifiable amino acids accounting for approximately 85% of total primary amines as estimated by fluorescamine. Thus it is more realistic to use a conversion factor of 1 ml of O_2 required for complete oxidation of a mixture of amino acids provided one assumes that rates of glycine influx are comparable to influx rates for other amino acid substrates. The table also includes the ambient free amino acid concentration (based on these assumptions) required to provide reduced carbon equivalent to oxygen consumption.

These data and calculations indicate that influx of free amino acids from ambient solution occurs at rates that are of the same order of magnitude as the requirement for reduced carbon to sustain oxidative metabolism; *i.e.* the process of transepidermal influx represents a potentially important source of supplementary nutrition for these animals. This conclusion is based on the assumption that bulk concentrations of free amino acids measured in interstitial water of the sediment habitat are a reasonable estimate of concentrations available to the animals. There is no direct information on this point, though Stephens (1975) has provided evidence that irrigation activity of infaunal annelids may increase local free amino acid concentration in interstitial water.

These data and calculations are not relevant to conditions during periods of anaerobiosis which infaunal worms may undergo periodically. Under such circumstances, influx rates for glycine in *Pareurythöe* and *Marphysa* decrease to 32 to 46% of aerobic rates. Requirements for reduced carbon will be considerably increased during periods of anaerobiosis. However, net influx continues from ambient concentrations in the range found in the habitat under anoxic conditions for up to 4 hr. Thus the process continues though its rate and overall contribution to metabolic requirements are both considerably reduced.

The presence of 2 mM KCN in the medium almost completely inhibits aerobic metabolism (oxygen consumption was reduced by 83%). This effect would be predicted based on the binding of CN^- to heme groups and the resulting interdiction

of electron transport. Thus the concentration used and the time of incubation employed were sufficient to force dependence on anaerobic metabolism. The resulting decrease in influx (40 to 45% of control values) agrees well with the decrease observed under anoxic conditions. Cyanide inhibition, both of influx and of oxygen consumption, were reversible; no mortality was encountered in these experiments. Thus this is additional evidence for the ability of these animals to tolerate periods of anaerobiosis. There is no evidence for a specific inhibitory effect of cyanide on the transepidermal transport system.

Mangum (1976) reviews respiratory adaptations of annelids. In her view irrigation of the burrow is one of the major adaptations to the sediment habitat in this group. Although data for *Marphysa* and *Pareurythöe* are not reported, it is reasonable to assume that they also irrigate their burrows. Mangum tabulates data comparing average P_{O_2} in the microhabitat (*i.e.* in the burrow) with P_{O_2} in the water column. The former ranges from 73 to 104 mm Hg when the overlying water column is essentially saturated. Thus the ability of the annelids examined in the present work to sustain normal rates of net influx at partial pressures of oxygen below saturation (Fig. 3) is of interest. There is little decline in influx rate until P_{O_2} drops below 20% of saturation. Since these estimates of the P_{O_2} actually encountered by the worms in the flow chamber are systematic overestimates (measured at the inflow end of the chamber), this suggests that the worms are functioning as aerobes at quite low P_{O_2}'s and hence at realistic levels of oxygen availability under normal circumstances. The depression of influx under anoxic conditions was greater in these experiments than was the case in the medium depletion experiments (15 to 20% rather than 32 to 46% of control values). The flow chamber was large in diameter compared to the worms. Under conditions of very low P_{O_2}, the worms typically coiled tightly. In contrast, medium depletion experiments were done with continuous gentle agitation by the gas stream (N_2 or air). It is possible that the greater depression of influx in the flow system is simply the result of this behavior and the reduction in surface exposed to the medium by the worms in the two situations.

The limitations of the use of oxygen consumption as a measure of metabolic requirements should be reemphasized. These limitations are based on the necessary assumption that metabolism is aerobic (clearly not always the case for annelids) and on the fact that requirements for organic material which must support growth and balance losses by other pathways do not enter the estimation process. It would be far more appropriate to undertake measurement of heat production (Pamatmat, 1978) and estimate minimum requirements on that basis; however, this has yet to be done for annelids. The calculations presented in Table II indicate that influx of naturally occurring free amino acids may provide a major input under aerobic circumstances. However, the significance of influx under anaerobic conditions will certainly be less, both by virtue of the reduction in rate and because of the increased requirements for substrate. In any case, the conclusion drawn from this work is that uptake of amino acids is a supplement. Both worms certainly have other feeding methods at their disposal.

The metabolism of facultatative anaerobic invertebrates is complex and involves a variety of end products (de Zwaan, Kluytmans, and Zandee, 1976). Some of the pathways demonstrated produce amino acids such as alanine and succinate

and these compounds might be expected to accumulate under anoxic conditions (Hochachka, Fields, and Mustafa, 1973). Though such reactions may well be involved in facultative anaerobiosis in annelids, the resulting amino acids are not perceptively lost to the medium in *Marphysa* and *Pareurythöe*; rates of efflux of primary amines are not increased under anoxic conditions (Figure 2).

The extent to which the two species examined in this work actually experience anoxic periods is probable very different. *Marphysa* was collected from a population in the intertidal from an area characterized by fine-grained sediment of high organic content. At low tide, animals almost certainly experience anoxia. *Pareurythöe* was collected from a shallow sub-tidal population in an area of coarser sediment. It is not clear why *Pareurythöe* would ever experience anoxia so long as burrow irrigation continues. Both species tolerate anoxic periods well and behave similarly in the present observations. Mangum (1976) suggests that the circulatory system in annelids may play a more important role in acquisition of oxygen from the environment than in distribution to deeper tissues; in many species deeper tissues are virtually avascular. Possibly the widespread tolerance of anoxia among annelids may reflect the fact that deeper structures may be poorly supplied with oxygen even in aerobic conditions.

Finally, the relation of this work to earlier reports of influx of ^{14}C-labeled substrates into marine polychaetes should be mentioned. Since it is now quite simple to examine influx and net flux of amino acids, further work in this area should include such examination. However, the coupling of influx as estimated radiochemically and net influx examined chemically has now been reported for several major groups of marine invertebrates and in each case examined, influx has been shown to be a close estimate of net flux provided concentrations are realistic and initial rates are compared. It seems increasingly likely that the early reports can be accepted provisionally as providing evidence for the existence of transport systems capable of net transport of substrate from the medium into the animal. The potential contributions of such transport will depend then on environmental availability of substrate and will no doubt vary among species. The wide distribution of transepidermal transport systems among marine invertebrates indicates that further work is desirable to describe the extent to which energy flow by this pathway may contribute to the trophic organization of marine communities.

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Summary

1. The effect of anoxia on influx and net flux of amino acids from dilute solutions into two species of marine polychaetes was studied.

2. Rates of influx and net flux correspond quite closely at ambient concentrations greater than 10 μM. Anoxic conditions, produced by incubating specimens of *Marphysa* and *Pareurythöe* in solutions containing 2 mM KCN or through which N_2 was bubbled, did not affect the tight correspondence between influx and net flux, though rates were reduced by approximately 50%.

3. The effect of P_{O_2} on influx and net flux was examined using a continuous

flow system. Influx and net influx remained at control rates down to P_{O_2}'s 10 to 20% of air saturation values.

4. Comparisons of rates of net flux to measured values of O_2 consumption indicate that these animals can acquire sufficient reduced carbon to account for their oxidative needs if their surfaces are exposed to amino acid levels on the order of 50 to 65 μM.

5. Primary amines in the interstitial water of sediments in the immediate vicinity of populations of these worms averaged between 123 and 131 μM.

6. *Marphysa* and *Pareurythöe* live in habitats that are relatively rich in amino acids, and they possess transport systems capable of the net accumulation of these compounds at rates sufficient to provide a significant supplement to other forms of feeding. The uptake process continues during periods of anoxia, though its rate and overall contribution to metabolic requirements are reduced.

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